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Bacteria–host interactions of *Salmonella* Paratyphi B dT$^+$ in poultry

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SUMMARY

In recent years, a dramatic increase in incidence of the dextro-rotatory tartrate-positive variant (dT$^+$) of *Salmonella enterica* subspecies *enterica* serovar Paratyphi B has been observed in poultry and poultry products. In the present study the interactions of this bacterium with the host were studied *in vivo* and *in vitro* in an attempt to explain the preferential association of this serotype with poultry. The ability of this organism to invade and multiply in chicken intestinal epithelial cells and the intracellular behaviour in chicken macrophages was studied *in vitro* using chicken cell lines. *In vivo* challenge experiments in specific pathogen-free chickens were carried out to determine the level of colonization of caeca and internal organs early after experimental infection. An *in vivo* trial with commercial broiler chickens, using a seeder model, was performed to determine whether *S.* Paratyphi B dT$^+$ could persist and spread in broilers until slaughter. *S.* Paratyphi B dT$^+$ invaded and multiplied in the chicken epithelial cell line and survived in a chicken macrophage cell line. The strain used colonized caeca and internal organs of chickens to a high extent 1 week after infection with a low-dose inoculum. Moreover, the strain was efficiently transmitted within a group of broilers and persisted until slaughter. It was concluded that *S.* Paratyphi B dT$^+$ was well adapted to poultry and therefore it is suggested that specific control measures against this serotype should be considered.

INTRODUCTION

Salmonella bacteria are the most widespread zoonotic pathogens in the world [1]. Poultry products constitute a major source of infection for humans [2], and over the years various serotypes have emerged in poultry populations. In the last two decades, *Salmonella enterica* subspecies *enterica* serovar Enteritidis has been the dominant serotype associated with poultry [3]. Recently, a high increase in incidence of the dextro-rotatory tartrate-positive variant (dT$^+$) of *S. enterica* subspecies *enterica* serovar Paratyphi B (*S.* Paratyphi B dT$^+$, formerly called *S.* Java) was detected in poultry and poultry products in Germany, as well as in neighbouring countries [4]. In these countries, a multiple drug-resistant clonal lineage of *S.* Paratyphi B dT$^+$ emerged and succesfully replaced those observed previously [5]. Non-European countries also report a high incidence of the dT$^+$ variant of *S.* Paratyphi B in poultry and the poultry

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environment [6]. Both d-tartrate-positive and d-tartrate-negative strains of *S.* Paratyphi B express similar pathogenic mechanisms, and appear not to be a major cause of enteric fever but primarily a cause of gastroenteritis in humans [7]. Although outbreaks of this *Salmonella* serotype in humans are sporadic, their recent significance should not be underestimated as they can cause severe disease [8–10].

The aim of this study was to observe the interactions between *S.* Paratyphi B dT^+ and a poultry host in order to better understand the present rise in the incidence of this serotype.

METHODS

Bacterial strain

Salmonella Paratyphi B dT^+ strain UG01, isolated from poultry carcass swabs in the slaughterhouse, was used in all experiments. The strain was serotyped by glass slide agglutination tests, resulting in the antigenic formula O4,[5]:Hb:H:2, specific for *S.* Paratyphi B. Fermentation of d-tartrate was tested by inoculation of the bacteria in Phenol Red tartrate agar (15 g agar, 10 g peptone, 10 g potassium tartrate, 5 g NaCl, 0·024 g Phenol Red/l water; pH 7·6). After overnight incubation at 37 °C, the media were yellow, indicating d-tartrate fermentation. The strain was resistant to sulphonamides, trimethoprim and nalidixic acid. The strain was grown in Luria–Bertoni (LB) medium for 6 h at 37 °C for inoculum preparation.

Chickens

Two *in vivo* trials were carried out. For the first trial, Lohmann Valo specific pathogen-free chickens (Lohmann, Cuxhaven, Germany) were used and were hatched and housed in isolation. For the second trial Ross commercial broilers were used and were housed in stables with concrete floors covered by wood shavings. All animals received irradiated feed (25 kGy γ-irradiation) and sterile water *ad libitum*, and were kept according to the guidelines of the ethical committee. After hatching and before challenge infection, cloacal swabs were taken from the chicks and checked for *Salmonella*.

In vivo trials

In the first *in vivo* trial groups of 10 SPF chickens were inoculated with 10^3 and 4×10^4 c.f.u. *S.* Paratyphi B dT^+ via the crop at hatching. After 7 days, the chickens were euthanized by intravenous embutramid (T61, Intervet, Mechelen, Belgium) injection and samples of caeca, liver and spleen were taken for bacteriological analysis.

In the second trial, 15 commercial 1-day-old broiler chickens were infected with 10^3 c.f.u. of *S.* Paratyphi B dT^+ and housed together with 20 non-infected chickens. Starting at 1 day after infection, cloacal swabs were taken at weekly intervals and bacteriologically analysed. At slaughter age (6 weeks), all animals were euthanized by intravenous embutramid injection and samples of caeca and spleen were taken for bacteriological analysis.

Bacteriological analysis

Bacteriological examinations of cloacal swabs were done by direct inoculation as well as by pre-enrichment/ enrichment. Direct inoculation was done by plating the swab on Brilliant Green agar (BGA; Oxoid, Basingstoke, UK). Pre-enrichment was performed in buffered peptone water (Oxoid) overnight at 37 °C, whereafter enrichment (overnight at 37 °C) was performed in tetrathionate Brilliant Green broth (Oxoid). Thereafter, a drop of the enrichment broth was plated on BGA.

In the animals of the first *in vivo* trial the number of c.f.u./g tissue of was determined by titration. In both trials, organs were pre-enriched/enriched. For titration, caeca, liver and spleen were homogenized with a Seward Stomacher® Lab Blender (Brinkman, Westbury, NY, USA) and 10-fold dilutions were made in buffered peptone water starting from 5-, 10- and 20-fold dilutions for caeca, liver and spleen respectively. For each dilution $6 \times 20\,\mu$l were inoculated on BGA. After overnight incubation (37 °C) the number of c.f.u./g tissue was determined. For samples which were negative after titration, pre-enrichment and enrichment were performed. Samples that were negative after titration but positive after *Salmonella* enrichment, were presumed to contain 5 (caeca), 10 (liver) or 20 c.f.u./g (spleen). The mean c.f.u./g tissue was calculated.

In vitro invasion of intestinal epithelial cells

The avian intestinal epithelial cell line DIV-1 was used in these experiments. These cells have the characteristics of differentiated intestinal epithelial cells [11]. DIV-1 cells were seeded in 96-well cell culture plates (Greiner, Frickenhausen, Germany) at a density of 5×10^5 cells/ml of Dulbecco's Modified Eagle Medium

(DMEM; Gibco–BRL, Paisley, UK) +10% foetal calf serum and 1% glutamine (without antibiotics) and grown overnight at 37 °C. Bacteria were grown for 6 h in LB medium at 37 °C, centrifuged and resuspended in DMEM. The number of c.f.u./ml was determined by plating $6 \times 20\,\mu$l of a tenfold dilution series of the suspension on BGA, and the plates were incubated overnight at 37 °C. The suspensions were stored at 4 °C until they were used in the assay. The bacterial suspension was diluted to a density of 5×10^6 c.f.u./ml DMEM. The growth medium of the cells was then replaced by 200 μl of the diluted bacterial suspensions. This was centrifuged for 10 min at 515 ***g***, whereafter the plates were incubated for 1 h at 37 °C and 5% CO_2. Then cells were rinsed three times with Hanks' Balanced Salt Solution (HBSS; Life Technologies, Paisley, UK), cell culture medium with gentamicin (50 μg/ml) was added and plates were incubated for 1, 2, 4, 6, 16, 20 and 24 h at 37 °C and 5% CO_2. At these time-points, cells were rinsed three times with PBS and lysed with 1% Triton X-100 in distilled water (Sigma, St. Louis, MO, USA). From this lysate, a tenfold dilution series was made. From each dilution, $6 \times 20\,\mu$l were inoculated on BGA, to determine the number of c.f.u./ml of *S.* Paratyphi B dT$^+$. The results are expressed as the percentage of intracellular bacteria, relative to the initial number of bacteria, brought on the cells.

In vitro survival in macrophages

The HD11 cell line, an avian macrophage cell line [12] was seeded at a density of 5×10^5 cells/ml in DMEM +10% foetal calf serum and 1% glutamine in 96-well plates and grown for 2 days. *Salmonella* Paratyphi B dT$^+$ bacteria were grown for 6 h in LB medium, their titre was determined as described above, and the bacteria were diluted in cell culture medium at a density of 5×10^6 c.f.u./ml. The cell medium was replaced by 200 μl of the bacterial suspension per well and the plates were centrifuged for 10 min at 1500 r.p.m. (37 °C). After 1 h of incubation, the cells were rinsed three times with DMEM. The medium was replaced with the above described cell culture medium with 50 μg/ml gentamicin. After 1, 2, 4, 6 and 24 h, cells were rinsed five times with HBSS without Ca/Mg. Then cells were lysed with Triton X-100 (1% in distilled water), and the number of intracellular bacteria was determined by plating $6 \times 20\,\mu$l of a tenfold dilution series of the lysate on BGA. The results are expressed as the percentage of intracellular bacteria, relative to the initial number of bacteria, brought on the cells.

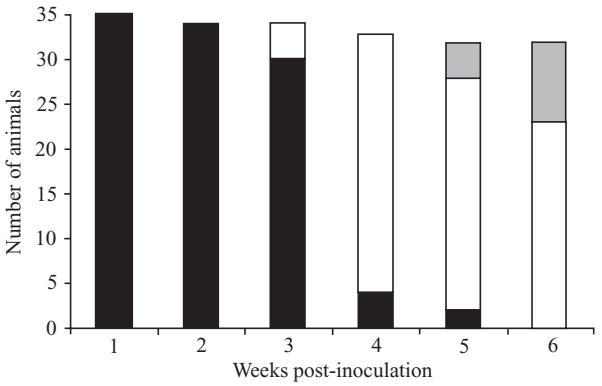

Fig. Number of cloacal swabs that were positive after direct inoculation (■), positive only after enrichment (□) or negative (▨) at different time-intervals after experimental infection or contact with experimentally infected chicks. Infection was done by inoculating 15 animals at hatch and housing them together from the time of infection with 20 non-infected animals. In weeks 2, 4 and 5 one animal died.

RESULTS

In vivo trials

All samples of caeca, liver and spleen were found to contain *Salmonella* bacteria after inoculation of SPF chickens at hatch with either 10^3 and 4×10^4 c.f.u. *S.* Paratyphi B dT$^+$. All caecal and spleen samples were found to be positive by titration, i.e. a number of c.f.u./g could be given. Six out of ten liver samples were only positive after enrichment in the group that was infected with 10^3 c.f.u., while this was the case for two animals after infection with 4×10^4 c.f.u. The mean values at 1 week after inoculation with 10^3 c.f.u. were 7·03 $\log_{10}$ c.f.u./g (±0·77), 2·06 $\log_{10}$ c.f.u./g (±1·44) and 3·84 $\log_{10}$ c.f.u./g (±1·08) for caeca, liver and spleen respectively. When the animals were infected with 4×10^4 c.f.u., the mean bacterial numbers per gram of organ were 6·74 $\log_{10}$ c.f.u./g (±0·62), 2·14 $\log_{10}$ c.f.u./g (±0·71) and 4·26 $\log_{10}$ c.f.u./g (±1·09) for caeca, liver and spleen.

After experimental infection of commercial broilers by a 'seeder' model, all animals had positive cloacal swabs after direct plating during the first 2 weeks post-infection (see Fig.). By week 3 post-infection, 4 out of 34 cloacal swabs were only positive after enrichment while the other 30 swabs were positive after direct plating. By week 4 post-challenge, there were already 29 out of 33 swabs that were only

Table. *Numbers of intracellular* Salmonella *Paratyphi B dT$^+$ bacteria in intestinal epithelial cells* (*DIV-1*) *and in avian macrophages* (*HD 11*) *at different time-intervals* (*in hours*) *after contact of* 5×10^6 *c.f.u.* S. *Paratyphi B dT$^+$ bacteria with the cells*

	Time-interval				
	1 h	2 h	4 h	6 h	24 h
DIV-1	9×10^3 c.f.u. ($\pm 5 \times 10^3$)	$2{\cdot}6 \times 10^4$ c.f.u. ($\pm 7 \times 10^3$)	$2{\cdot}9 \times 10^4$ c.f.u. ($\pm 6 \times 10^3$)	4×10^4 c.f.u. ($\pm 2{\cdot}5 \times 10^4$)	$8{\cdot}5 \times 10^5$ c.f.u. ($\pm 1{\cdot}8 \times 10^5$)
HD 11	$2{\cdot}2 \times 10^5$ c.f.u. ($\pm 9{\cdot}5 \times 10^4$)	$3{\cdot}5 \times 10^5$ c.f.u. ($\pm 1{\cdot}1 \times 10^5$)	$4{\cdot}7 \times 10^5$ c.f.u. ($\pm 5{\cdot}3 \times 10^4$)	$2{\cdot}3 \times 10^5$ c.f.u. ($\pm 7{\cdot}8 \times 10^4$)	$8{\cdot}5 \times 10^5$ c.f.u. ($\pm 2{\cdot}4 \times 10^5$)

positive after enrichment. In weeks 5 and 6 post-infection, the number of swabs, positive after direct plating decreased further (2/32 and 0/32 respectively) while the number of negative animals increased (4/32 and 9/32 respectively).

At slaughter, all caecal samples were positive after enrichment, while 12 out of 32 spleen samples were positive after enrichment.

In vitro experiments

There were 9×10^3 c.f.u. of *S.* Paratyphi B dT$^+$ bacteria detected 1 h after 5×10^6 c.f.u. of *S.* Paratyphi B dT$^+$ bacteria were brought on the cells. The number of intracellular bacteria in the cells increased over a 24-h period, reaching approximately $8{\cdot}5 \times 10^5$ c.f.u. of *S.* Paratyphi B dT$^+$ bacteria at 24 h after initial invasion of the cells (Table). This implies an intracellular multiplicity of approximately 95 times during 24 h.

At 1 h post-inoculation of HD11 macrophage cells with 5×10^6 c.f.u. of *S.* Paratyphi B dT$^+$, $2{\cdot}2 \times 10^5$ c.f.u. of *S.* Paratyphi B dT$^+$ could be detected intracellularly. After 2 and 4 h, the bacterial number in the cells increased, while a decrease was detected after 6 h (Table). After 24 h, $8{\cdot}5 \times 10^5$ c.f.u. of intracellular *S.* Paratyphi B dT$^+$ bacteria were detected, which is 3·76 times more bacteria compared to 1 h after inoculation.

DISCUSSION

From the present study it was concluded that *S.* Paratyphi B dT$^+$ has the ability to colonize caeca and internal organs of young chickens to a high extent and to persist in commercial broilers until slaughter. Indeed, infection of specific pathogen-free 1-day-old chicks with low doses of *S.* Paratyphi B dT$^+$ resulted in a high number of these bacteria in the caeca 1 week after inoculation. Moreover, the strain efficiently colonized internal organs. The extent of colonization of caeca and internal organs by *S.* Paratyphi B dT$^+$ was of the same magnitude as after infection of 1-day-old chicks with equal doses of a virulent *S.* Enteritidis strain [13, 14]. In commercial broilers, the strain was transmitted in a group of chickens with high efficiency. After an experimental infection of 15 birds which were housed together with 20 non-infected birds, all birds excreted the *Salmonella* strain 1 week after challenge. All animals had *S.* Paratyphi B dT$^+$-positive caeca at 6 weeks post-inoculation. These results imply that the *S.* Paratyphi B dT$^+$ strain has the potential to rapidly spread between chickens and to persist in chickens until slaughter age. Thus there can be a risk for contamination of food products. *Salmonella* strains, detected in hatcheries or farms can be found on fully processed chicken carcasses, dependent on the serovar and the strain [15, 16]. Certain serotypes and strains can be rapidly eliminated by chickens so that they no longer carry the strains when they enter the slaughterhouse. Other serotypes and strains, in contrast, can be carried within the chicken from hatch until slaughter [17, 18]. Based on our results it can be concluded that the emerging pathogen *S.* Paratyphi B dT$^+$ is a serotype that is persistent in the chicken.

In the present study, it was shown that persistence might very well be due to the ability of the strain to multiply in chicken enterocytes and macrophages. The *S.* Paratyphi B dT$^+$ strain invades intestinal epithelial cells and multiplies within these cells to a high extent. Indeed, 24 h after entry of the bacteria in the cells there are 95 times more bacteria present in the cells. This can be explained only by multiplication in the cell since *Salmonella* spp. are not known to be able to spread laterally in cell culture systems, in contrast to *Listeria* [19]. Multiplication in intestinal epithelial cells can partly explain the high caecal counts of *Salmonella* bacteria. Moreover, *Salmonella* bacteria are known to reach the lamina propria where

they are taken up by macrophages. The *in vitro* results show that *S.* Paratyphi B dT$^+$ can survive within macrophages and even multiply within these cells. It is clear that in this way colonization of internal organs can be efficient. Nevertheless the degree of invasiveness in epithelial cells and intracellular survival are not always correlated with virulence of *Salmonella* [20, 21].

In conclusion, *S.* Paratyphi B dT$^+$ is able to spread within a group of chickens and to persist in broiler chickens until slaughter age. This serotype has a high capacity to multiply in chicken enterocytes and macrophages. This implies a risk for transfer of this emerging pathogen in the food chain. This serotype should thus be considered as a threat to public health. It is therefore suggested that specific control measures against this serotype should be considered.

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